MEMORANDUM

SUBJECT: Construct Hazard Analysis for TERA R18-01

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DATE: November 15, 2018

**********[redacted]**********

I. Introduction

EPA has received a TSCA Environmental Release Application (TERA) from Arizona State University on behalf of the Producing Algae for Coproducts and Energy (PACE) Consortium to test one different intergeneric eukaryotic algal constructs in open ponds to gather data for commercialization. The subject strain of this non-CBI TERA R-18-0001 is *Chlorella sorokiniana* PACE_Cs1412_SNRK2 (from now on known as Cs1412_SNRK2). This TERA application is similar to one received last year, TERA [redacted] which is based on the same species *C. sorokiniana*. The submitter identifies the parental organism and recipient alga as *C. sorokiniana* DOE1412. The Subject strain was developed with molecular biology approaches.

The purpose of this genetic modification and subsequent field experiment is to:

1) evaluate the translatability of genetically modified phenotypes from a lab to an outdoor setting;
2) compare the subject and recipient microorganisms when cultivated in outdoor miniponds with respect to their ability to produce biomass; and
1) identify any increase or decrease in biomass productivity under biotic (bacteria, predators) and abiotic (diurnal temp and light) stressors from the environment

A native *C. sorokiniana* promoter and terminator for the *psaD* gene coding for subunit II of photosystem I reaction center is used to regulate and drive the expression of an intergeneric sucrose non-fermenting (SNF) related kinase (*SNRK2*) gene in the Subject strain Cs1412_SNRK2. The SNRK2 enzyme is being utilized to improve photosynthetic efficiency and biomass in the recipient organism.

In addition, an actin promoter/terminator pair, which is endogenous to the recipient microorganism, is used to regulate and drive the expression of the intergeneric *Streptoalloteichus hindustanus* Sh *ble* gene, which confers zeocin resistance.

The foreign genes are expected to integrate within the nuclear genome and the protein products of both genes are expressed in the cytoplasm of the Subject microorganism.

II. Genetic Construction of the Microorganisms

The submitter identifies the parental and recipient organism as *Chlorella sorokiniana* DOE1412. This strain was isolated from the field by Juergen Polle in 2013 (UTEX website, accessed 09/2018) and deposited to the CUNY collection. Subsequently, the National Alliance for Advanced Biofuels and Bio-Products (NAABB) consortium, after a screening process, has made 30 of their best performing strains, including DOE1412, available to the public through UTEX. These UTEX strains have been well characterized by DOE for lipid production and growth kinetics. UTEX and DOE, describe the strain as a high temperature freshwater strain (cold-sensitive) with a maximum growth temperature of 42^{o}C. The strain is also referenced as DOE1412, NAABB 1412, NAABB 2412, and UTEX B 3016.

The genes in the Subject strain Cs1412_SNRK2 were cloned into the PACE *Chlorella* Zeocin plasmid vector. The PACE vector was developed by researchers at the New Mexico Consortium to introduce genes of interest into *Chlorella sp.* and is built on the *E. coli* vector backbone from plasmid pSL18. It contains:

1) an *E. coli* plasmid origin of replication;
2) an *E. coli* plasmid ampicillin resistance (AmpR) gene for propagation in *E. coli*;
3) an empty multiple cloning site (MCS) for insertion of the gene of interest;
4) the psaD promoter/terminator pair flanking the MCS. This promoter was chosen due to its relatively high expression as a native photosynthesis-related gene promoter in *C. sorokiniana*; and
5) an intergeneric *Streptoalloteichus hindustanus* Sh *ble* gene, which confers zeocin (ZeoR) resistance, under the control of th*e C. sorokiniana* actin promoter/terminator

The critical components of each construct that are expected to integrate into the nuclear genome of the subject microorganism are the *psaD* promoter sequence, the intergeneric gene coding sequence (*SNRK2*), the *psaD* terminator along with the selection marker, which integrates as actin promoter, ZeoR coding sequence for zeocin, and actin terminator. For more details, refer to the Genetic Construction Report by Cameron 2018. Note that the SNRK2_PACE_Chlorella_plasmid was linearized before transformation by a single digest that disrupted the AmpR gene (*bla*), therefore a full *bla* sequence was not integrated into the genome of the submission strain. During translation, SNRK2 is expressed in the cytosol.

The *SNRK2* intergeneric gene used to develop the Subject strain in this TERA was derived from a *Picochlorum soloecismus* strain, a genus of green algae in the class Trebouxiophyceae. *Picochlorum soloecismus* is a halotolerant, fast-growing and moderate lipid producing microalga that has been evaluated as a renewable feedstock for biofuel production by the DOE (Gonzalez-Esquer et al., 2018).

SNRK2 is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes. Per the submitter, the SNRK2 enzyme plays a critical role in Arabidopsis plants to regulate the energy metabolism. The over-expression of *SNRK2* gene in Arabidopsis increases

sucrose synthesis, starch synthesis and leaf growth. They expect overexpression of *SNRK2* gene would improve starch accumulation and growth in *Chlorella* cells (see section B.4.f. and B.4.c in the submitted TERA). The gene was synthesized in its native state (only the coding regions) without codon optimization and cloned into the PACE Chlorella plasmid vector. The regulatory elements used to express the SNRK gene are the *psaD* (a photosynthesis-related gene) and actin promoters and terminators, both of which are endogenous to the recipient microorganism.

A summary of the intergeneric genes in the Subject strain is in Table 1 below.

Table 1. Genetic and biochemical details provided for the intergeneric genes used in constructing the subject strain.

Recipient Strains	Gene	Source Organism(s)	Function	Promoter	Terminator	Artif Synth.?	Codon-Opt.?
C. sorokiniana DOE 1412	SNRK 2 [encodes sucrose non-fermenting related kinase] Gonzalez et al., 2018.	*Picochlorum soloecismus*	Regulate energy metabolism	*psaD* from *C. sorokiniana*	*psaD* from *C. sorokiniana*	Yes By GeneWiz (p.9, 15)	**No** (p.9, 15)
C. sorokiniana DOE 1412	Sh *ble* (encodes resistance to members of the BLM antibiotic family, including zeocin (a.k.a., phleomycin D1 per PubChem)	*Strepto-alloteichus hindustanus* ATCC 31158	Small, acidic, non-enzymatic protein that binds to some members of the bleomycin (BLM) antibiotic family with high affinity. BLMs are glycopeptide antibiotics that cleave DNA when activated with ferrous (Fe^{2+}) ions and oxygen. When bound to Sh ble protein, they cannot be activated toward this purpose. Specifically confers resistance to tallysomycin, zeocin (phleomycin D1), and bleomycin. (Drocourt et al. 1990; Chankova *et al.* 2007, Gatignol *et al.* 1988, Miyazaki et al. 2009	Actin promoter from *C. sorokiniana*	Actin terminator from *C. sorokiniana*	Yes By GeneWiz (p. 15)	Yes (P.10)

III. Hazards of the Introduced Genetic Material

The subject strain is engineered to carry resistance to the antibiotic Zeocin (phleomycin D1, zeomycin) via the intergeneric gene *Sh ble* from *Streptoalloteichus hindustanus* (Kim, et al. 2013). The antibiotic zeocin™ is a laboratory chemical that is not approved, and thus not used, in human or animal medicine. Zeocin is the commercial name for a special formulation of phleomycin D1 (Ceozin), a glycopeptide antibiotic. It is a member of the glycopeptide antibiotic

of the bleomycin family of antibiotics. Resistance to this antibiotic is encoded by the *Sh ble* gene (http://www.genaxxon.com/docs/pdf/zeocin_data.pdf). According to this website, there is no cross resistance of zeocin with other antibiotics such as G418 (neomycin/geneticin), hygromycin B, blasticidin S, or puromycin. However, it is unknown if zeocin also encodes resistance to bleomycin and phleomycin. Although these latter two antibiotics are inhibitory to a number of bacteria, they are used clinically for their antitumor activity as they reduce the growth of cancer cells. Thus, it is possible that there would be little concern for their use in genetically engineered microorganisms as these antibiotics appear not to be used to treat infections in humans or animals. According to the submitter, the zeocin antibiotic will not be used in the field under any circumstances. During the course of the field experiment, it will be necessary to inactivate the subject microorganisms. The submitter plans to use bleach and/or autoclave the algal strains as a way to inactivate cultures and biomass.

With regard to altered functions (differing substrate ranges for the subject enzymes, changes in enzyme kinetics, folding, and other protein changes as a result of using synthetic sequences as opposed to sequences obtained directly from the intergeneric donor organisms), the likelihood for this to occur is considered low. Note that the transcription of DNA beyond the insertions is limited by terminator sequences.

The subject microorganism, which is taxonomically the same as its parental strain, *C. sorokiniana,* is not likely to pose any risk to human health and mammalian species. *Chlorella sp.* are generally regarded as safe (GRAS) for human consumption according to the FDA (Bagchi *et al.*, 2016; Roberts *et al.* 2016). In humans *Chlorella sp.* supplements have shown beneficial effects including improved immune responses, improved healing of the small intestine epithelium, antioxidant action and even antitumoral effects (Ramirez-Romero *et al.*, 2010). Neither *C. sorokiniana* nor the production microorganisms produce or contain any xenobiotic contaminants. There is limited evidence in the literature of *C. sorokiniana* producing toxins, antibiotics, xenobiotic or degradative gene products. Some studies have discovered that *Chlorella* produces an antibiotic, chlorellin, which is a growth-inhibiting substance against itself, other algae, gram positive bacteria, and gram negative bacteria (Ryther 1954, Pratt 1944). The submitter carried out a homology search of the subject microorganism's intergeneric genes against known allergens, and did not observe any significant results with identity matches greater than 22%.

However, *C. sorokiniana* is considered a weak allergen and may induce asthma to certain groups of patients (Tiberg *et al.*, 1995, Ng *et al.*, 1994). There have been reported incidents of *Chlorella sp.* infections in compromised tissues of humans and animals exposed to contaminated waters, but these algal infections are rare (Yu *et al.*, 2009, Ramirez-Romero *et al.*, 2010). Further studies are needed on chlorellosis to determine the pathogenicity and virulence of *Chlorella* in humans and animals (Krcmery Jr *et al*., 2000).

Chlorella sp. has been found as part of the freshwater plankton in rivers, ponds, and lakes, as well as in marine and edaphic habitats around the world (Bock *et al.,* 2011; Ratha *et al.,* 2012). Like many other algae, *Chlorella* plays an important role in the aquatic food web as a primary producer and food source for higher trophic levels. No enhanced potential adverse effects for the two strains are expected, relative to those noted above for the recipient strain.

IV. Potential for Gene Instability and Transfer

The intergeneric genes are expected to be stably integrated into the nuclear genome. The stability of the DNA can be assessed by PCR and qPCR after multiple generations of growth. While the studies with the subject microorganisms have been relatively short, no loss of introduced DNA has been observed in previously created microorganisms after the initial transformation event. In the previous TERA [redacted], to avoid such losses, [redacted] as described in the "Backcrossing *Chlorella sorokiniana* SOP" to avoid any reversion to wildtype genetic background. According to the submitter, for this TERA R-18-01, no backcrosses were carried out since PCR/RT-PCR positive amplicons of SNRK in the transgenic lines has been conserved for two years without the need to carry out a backcross. This demonstrates that the SNRK overexpression is stable in *Chlorella.*

Although there is a possibility for gene transfer through mating with wild *C. sorokiniana* strains, PubMed searches carried out by the submitter on *C. Sorokiniana** gene transfer* genetic exchange* did not produce results indicating the prevalence of gene exchange of the recipient microorganism in natural populations. Prior to initiating the field trial experiments, the submitter will sample soil from the field test site to determine what organisms may be present in the microbial community within the soil. The submitter will perform a similar analysis on the local surface water from the canal system to the east of the Polytechnic campus.

Horizontal gene transfer among bacteria is widespread and is responsible for acquisition of a myriad of traits in bacteria such as antibiotic resistance, xenobiotic degradation pathways, and even pathogenesis (McClung, 2013). Not nearly as much is known regarding horizontal gene transfer in eukaryotes. It has been thought that the barriers to horizontal gene transfer in bacteria are even worse in eukaryotic organisms because of the complexities in their transcription and translation mechanisms (Raymond and Blankenship, 2003). However, from evolutionary analyses, horizontal gene transfer in eukaryotes is known to have occurred. For example, in evolutionary times, it was a primary endosymbiotic event of a cyanobacterium being engulfed that gave rise to the photosynthetic plastid in the common ancestor of the Plantae, such as red and green algae and higher plants (Chan *et al*., 2012). Likewise, the mitochondria arose from the endosymbiosis and subsequent genetic integration of an alpha-proteobacterium (Keeling and Palmer, 2008; Timmis *et al*, 2004). In addition, investigations of the *Chlorella* genome, specifically *Chlorella variabilis*, suggest the ability for *Chlorella* to produce chitinous cell walls as a result of genetic material uptake from algal viruses, prokaryotes, and fungi (Blanc *et al,* 2010). Eckardt *et al*., 2010 hypothesized that the *Chlorella* chitin metabolism genes could have been acquired via horizontal gene transfer from viruses. There are other episodes of lateral gene transfer in eukaryotes, such as the phagocytosis by the sea slug *Elysia chlorotica* of the alga *Vaucheria litorea.* The photosynthetic sea slug maintains the algal plastids which continue to photosynthesize for months within the slug (Rumpho *et al*., 2008).

Very little is known about horizontal gene transfer from one algal species to another. However, there is also evolutionary evidence for horizontal gene transfer in algae. Archibald *et al*. (2003) found that of the 78 plastid-targeted proteins in the chlorarachniophyte alga *Bigelowiella natans*, approximately 21% of them had probably been acquired from other organisms including streptophyte algae, red algae (or algae with red algal endosymbionts), and bacteria. However, in the green alga *Chlamydomonas reinhardtii,* the homologous genes did not show any evidence of lateral gene transfer. It was suggested that this may be because this green alga is solely autotrophic whereas the *Bigelowiella* is both photosynthetic and phagotrophic. Another

instance of potential lateral gene transfer having occurred in algae is the work presented by Raymond and Kim (2012). They found the presence of ice-binding proteins in sea ice diatoms that apparently were essential for their survival in the ice. These protein genes were completely incongruent with algal phylogeny, and the best matches were all bacterial genes. Like bacterial genes, they did not contain introns. There is one example of horizontal gene transfer from an alga to its DNA virus. By phylogenetic analysis, Monier *et al*. (2013) demonstrated that the transfer of an entire metabolic pathway, consisting of seven genes involved in the sphingolipid biosynthesis, from the eukaryotic alga *Emiliania huxleyi* and its large DNA virus known as EhV had occurred.

There is no information in the literature on horizontal gene transfer specifically with *Scenedesmus* sp. It is unlikely that the fatty acid synthesis genes would be transferred to other green algae as they do not provide for any selective advantage in the environment. In addition, as previously mentioned, according to Radakovits *et al*. (2010) the increased production of fatty acids may result in a reduction of cell division and microalgal proliferation.

There is the potential for vertical gene transfer through sexual reproduction. As previously mentioned, sexual cells, gametes, have been observed for *Scenedesmus obliquus* (Trainor, 1998). However, it was only under extremely specialized laboratory conditions that gametogenesis could be induced (Trainor, 1998). Other laboratories were apparently unable to reproduce the results. Although the presence of gametes has been observed in nature, Trainor (1998) said that cell fusion or zygote formation has not been observed. It is unlikely that sexual reproduction would contribute to gene transfer to other organisms.

In addition, it is possible that the recipient and thus the subject microorganisms are capable of dispersion through airborne transfer, suggesting that gene transfer may occur away from the testing site. Genitsaris *et al*. (2011) did a comprehensive review of studies in the published literature on airborne algae. They summarized that the most frequently occurring algae isolated from aerosols were *Chlorella, Scenedesmus, Chlorococcum,* and *Klebsormidium,* and the cyanobacterium *Lyngbya.* These were found in more than 40% of the sites that had been sampled by various researchers in their aero-algae studies. Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma *et al*., 2007). Desiccation, the equilibration of an organism to the relative humidity of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten, 2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker *et al*. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1960) who reported survival of only four hours with desiccation stress. Ehresmann and Hatch (1975) studied the effect of relative humidity (RH) on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19,40,60,80, and 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity. In an earlier study Schlichting (1961) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So

perhaps, the gradual air-drying of soil samples as in Parker *et al*. (1969) did not result in death of the microorganisms. Slow desiccation to an air-dried state was shown to facilitate faster growth recovery of the *Scenedesmus dimorphus* and the marine alga *Nannochloropsis* than fast desiccation, although both strains showed significant growth inhibition after desiccation (Anadarajah *et al*., 2011).

V. Conclusions

C. sorokiniana is not known to produce any toxins that might be harmful to humans, animals, or plants. The modifications made to the recipient microorganism involve introducing the SNRK2 from *Picochlorum soloecismus.* The modifications are not expected to introduce any other phenotypic change in the recipient microorganism and will not impart or enhance any harmful traits beyond what may be present in the recipient strain. The submitter plans to evaluate the translatability of genetically modified phenotypes from a lab to an outdoor setting, compare the Subject and Recipient microorganisms' abilities to produce biomass under biotic and abiotic stress from the environment, identify any increase or decrease in biomass productivity/growth rates under biotic and abiotic stress from the environment, compare the resistance of the Subject strain to biotic (bacterial) and abiotic (diurnal temperature and solar insolation) factors versus the Recipient (wild-type) strain, and understand how microalgae migrate and affect natural plankton communities. The proposed field test poses low concern for humans and the environment as a result of the introduced DNA. Due to uncertainties associated with the hazard and gene transfer information, the testing proposed should be pursued to inform future larger scale releases.

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